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Research Article

Marine sponge depsipeptide increases gap junction length in HTC cells transfected with Cx43–GFP

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Abstract

Connexins are membrane proteins that form GJ (gap junction) channels between adjacent cells. Cx43 (connexin 43), the most widely expressed member of the connexin family, has a rapid turnover rate, and its degradation involves both the lysosomal and ubiquitin–proteasome pathway. The goal of this work was to study the effects of geodiamolides, natural peptides from marine sponge that normally are involved with microfilament disruption, on connexin assembly or degradation in the plasma membrane. HTC (hepatocarcinoma cells) expressing Cx43–GFP (green fluorescent protein) were submitted to treatment with 200 nM geodiamolides A, B, H and I for 2 and 4 h. Microfilament distribution and the presence and size of GJ plaques were evaluated by laser scanning confocal microscopy. Among the four peptides tested, only Geo H (geodiamolide H) statistically enhanced the length of GJ plaques. Geodiamolide A also showed activity in the GJ plaque size; however, its effect was less pronounced. Treatment with Geo H could interfere with the delivery of connexins to the degradation structures, similar to proteasomal pathways, keeping the connexins assembled and accumulating GJ plaques. Further experiments, with the cells treated with Geo H, using the fungal antibiotic BFA (brefeldin A), were performed in order to uncouple events leading to GJ assembly from those related to GJ removal, since BFA is known to block protein trafficking within a fused ER (endoplasmic reticulum)/Golgi compartment. GJ plaques were drastically reduced after BFA/Geo H treatment, thus indicating that Geo H affects mainly the delivery pathway of Cx43 protein.

Keywords: gap junction; geodiamolide; green fluorescent protein (GFP); hepatocarcinoma; marine sponge

1. Introduction

GJs (gap junctions) are communicating junctions that are present in most cells in animal tissue. The GJ channels are formed by four-pass transmembrane proteins, connexins, six of which assemble to form a channel, a connexon. When the connexons in the plasma membranes of two cells in contact are aligned, they form a continuous aqueous channel that connects two cell interiors. Thus, clusters of connexons allow molecules smaller than about 1000 Da to pass directly from the inside of one cell to the inside of the next. Cells connected by GJs share many of their inorganic ions and other small molecules and are, therefore, chemically and electrically coupled. GJs are important in coordinating the activities of electrically active cells, and they have a co-ordinating role in other groups of cells as well (Beyer, 1993; Saez et al., 2003).

The synthesis, assembly and turnover of GJ channels appear to follow the general secretory pathway for membrane proteins. Since there are different types of connexins, the connexin polypeptide subunits can assemble homo- or hetero-oligomeric connexons. The ability to form homotypic and heterotypic channels that consist of two identical or two different connexons, respectively, adds even greater versatility to the functional modulation of GJ channels (Yeager et al., 1998).

More than 20 connexin isoforms encoded by different genes have been described in the human genome, allowing the synthesis of a large number of channels with different functional properties. Cx43 (connexin 43) is the most widely expressed connexin family member (Butkevich et al., 2004), with 21 connexin isoforms described in humans (Söhl and Willecke, 2004). Cx43 trafficking, assembly and turnover are regulated by multiple mechanisms, including those mediated by integrins, by extracellular matrix and by the cytoskeleton (Guo et al., 2003). According to Giepmans et al. (2001), the Cx43 tail binds directly to tubulin, suggesting that, in addition to its well-established role as a channel-forming protein, Cx43 can anchor microtubule distal ends to GJs and thereby might influence the properties of microtubules in contacted cells.

Actin microfilaments are involved in GJ maintenance. Wang and Rose (1995) demonstrated that the mechanism for Cx43 channel clustering depends on intracellular cAMP and intact microfilaments. Thus, plaque formation may be regulated by other proteins, including PKA (protein kinase A) and actin. More recently, drebrin was described as a new actin-binding protein. Drebrin has also been shown to bind to the CT (C-terminus) of Cx43, and this suggests that Cx43 is possibly bridged to microfilaments. Silencing of drebrin resulted in reduced GJIC (GJ intercellular communication), accompanied by the internalization of Cx43, suggesting a role

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Abbreviations used: ANOVA, analysis of variance; BFA, brefeldin A; Cx43, connexin 43; Geo, geodiamolide; GJ, gap junction; HTC, hepatocarcinoma cells; PKA, protein kinase A.

for drebrin in maintaining GJs at the cell surface (Butkevich et al., 2004; Laird, 2006).

In two previous studies by our group, we investigated the effects of the geodiamolides (A, B, H and I), depsipeptides isolated from the marine sponge *Geodia corticostylifera*, in cultured cells. These peptides have antiproliferative activity on breast cancer cells due to microfilament disruption (Rangel et al., 2006). Further experiments with Geo H (geodiamolide H) and breast cell cultures (normal and tumoral) growth in a 3D environment showed that the peptide induced striking phenotypic modifications in the Hs578T cell line (a poorly differentiated and aggressive cell line) and disruption of the actin cytoskeleton. The peptide seemed to revert Hs578T malignant phenotype and impaired the migration and invasive behaviour of this cell line (Freitas et al., 2008). Interestingly, in both studies, the geodiamolides were not cytotoxic for normal cell lines and did not affect the normal distribution pattern of the microfilaments.

Considering that microfilaments may be involved on GJC (GJ communication) maintenance at the cell membrane and that geodiamolides disrupt actin microfilaments of cancer cell lines in *in vitro*, our objective was to investigate if the geodiamolides A, B, H and I can induce alterations on GJ stability in HTC (hepatocarcinoma cells) transfected with Cx43–GFP (green fluorescent protein).

2. Materials and methods

2.1. Cell culture and transfection conditions

HTC cells (derived from rat hepatocarcinoma) were cultured in DMEM (Dulbecco's modified Eagle's minimum essential medium; Sigma), supplemented with 5% FBS (fetal bovine serum, Cultilab). One day before the transfection, the cells were plated in 35-mm Petri dishes at a density of 5×10^4. The transfections were performed with Lipofectin reagent according to manufacturer's specifications (Invitrogen-Life Technologies). Transfected cells expressing Cx43–GPF genes were called HTC–Cx43–GPF.

2.2. Plasmid construction

Cx43 cDNA (GenBank® no. NM24392) was isolated from *Rattus norvegicus* (clone G2A) containing the full-length sequence and used directly as a template on polymerization reactions as described by Ionta et al. (2009). After amplification and purification, the corresponding Cx43 fragment (~1.1 kb) was cleaved (BamHI), purified and linked to the plasmid pEGFP-N1 (Clontech; EGFP is enhanced GFP), which was previously cleaved by BglII and SmaI (BglII and BamHI have compatible extremities). The recombinant plasmid was verified by DNA sequencing on an ABI-PRISM 377 (Applied Biosystems) and was called pCx43–EGFP.

2.3. Treatment

The cells were cultured on coverslips into 35-mm dishes at 5×10^4 cells/plate. The cells were treated when cell-to-cell contacts were well established (48 h after plating). The medium containing geodiamolides A, B, H or I at 200 nM concentration was added for 2 or 4 h. To study the Cx43 delivery pathway, some samples were treated with brefeldin A (BFA) (5 μg/ml) in combination or not with Geo H (200 nM) for 2 or 4 h.

2.4. Fluorescence staining and confocal analysis

After the treatment, the cells were fixed (formaldehyde, 3.7% in PBS), the plasma membrane was permeabilized (Triton X-100 – 0.5%), and microfilaments were labelled with phalloidin–TRITC (tetramethylrhodamine β-isothiocyanate; Sigma) for 20 min. The nuclei were stained with propidium iodide (10 μg/ml) or TOPRO-3 (Molecular Probes), depending on the cytological preparation. The analyses were performed using a confocal laser scanning microscope (LSM 510, Zeiss). The lasers [argon (488 nm), helium–neon1 (543 nm) and helium–neon 2 (633nm)] were connected to an inverted fluorescence microscope (Axiovert 100M, Zeiss).

2.5. GJs measurements

The GJ plaques formed between two adjacent HTC cells had their length measured in control experiments and after treatments with geodiamolides (200 nM). Three replicas of each condition were analysed by confocal laser scanning microscopy, and the images of at least ten different fields of each one were recorded. The measurements were performed using the software Zeiss LSM Image Browser (v. 4.0) and the tool *measure*, with a ×20000 (optical+digital zoom) image amplification on the screen (1 μm=2 cm). The number of GJ plaques measured varied from 72 to 102, depending on the condition, and represented all GJ plaques formed in these laminas.

2.6. Statistical analysis

The GJs formed in HTC cells had their lengths measured in control experiments and after treatment with geodiamolides. The measurements were performed using the software Zeiss LSM Image Browser (v. 4.0). At least 72 GJ were counted for each condition. Mean and S.E.M. calculations, and ANOVA (analysis of variance) followed by the Newman–Keuls multiple comparison test were performed using GraphPad Prism software (v. 4.0). A 5% significance level was adopted in this analysis.

3. Results

The results obtained after treatments with geodiamolides showed that both geodiamolides A and H (200 nM concentration for 2 h) increased the length of GJ plaques in HTC–Cx43–GFP cells. In the control cells, Cx43 was widely distributed in the cytoplasm with some punctal staining at the plasma membrane. In contrast, the cells treated with geodiamolides A and H exhibited Cx43, preferentially localized at the cell surface, forming GJ plaques. Images obtained by confocal microscope showed larger GJ plaques in treated cells (Figure 1). The microfilament network was not disrupted when geodiamolides were used at 200 nM

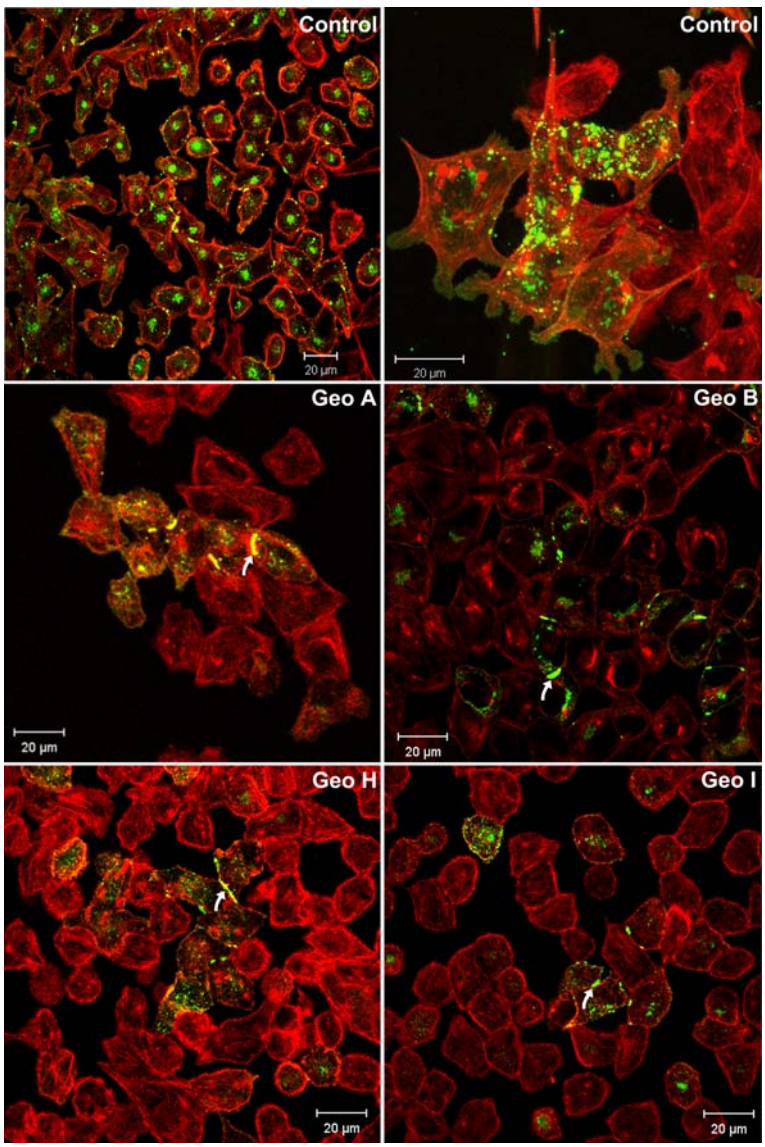

Figure 1 **Laser scanning confocal microscopy images of HTC–Cx43–GFP cells**
Microfilaments were stained with phalloidin–TRITC (red) and Cx43 is shown in green. Geodiamolides A, B, H and I were used at 200 nM concentration for 2 h. GJs plaques (arrows) are more visible in cultures treated with geodiamolides, and actin filament distribution was not altered.

concentration for 2 h. The actin filaments were distributed in the cytoplasm organizing some stress fibres and were also concentrated in the cellular cortex (Figure 1).

By measuring the length of GJ plaques in different images, we found that Geo H induced a significant increase (38.4%) in the length of GJ plaques. Geodiamolide A also increased the length of GJ plaques (25.4%); however, the difference was not statistically significant (Figure 2). Considering that Geo H increased significantly the length of GJ plaques without disrupting the microfilament network, we investigated if these effects also would be observed if the time of treatment was extended to 4 h. Figure 3 shows cells treated with Geo H (200 nM, 4 h), where GJ plaques can be observed on cell-to-cell contact areas. The actin filaments were distributed in the cellular cortex and in the cytoplasm, demonstrating that there was no microfilament disruption. The treatment did not alter the cellular features; the cells exhibited polyhedric morphology, and the cell-to-cell contacts were established. Although the 200 nM Geo H for 2 or 4 h induced larger GJ plaques without changing the actin filaments organization, when higher geodiamolide concentration was used (e.g. 400 nM), we observed drastic microfilament disruption. Under these conditions, the cells became rounded, and GJ plaques were absent (data not shown).

Further treatments were performed using BFA in combination or not with Geo H at 200 nM for 2 or 4 h. The results showed that GJ plaque formation was strongly inhibited by BFA. Cx43 was observed only in the cytoplasm, demonstrating that the delivery pathway of Cx43 protein to membrane was inhibited. When the

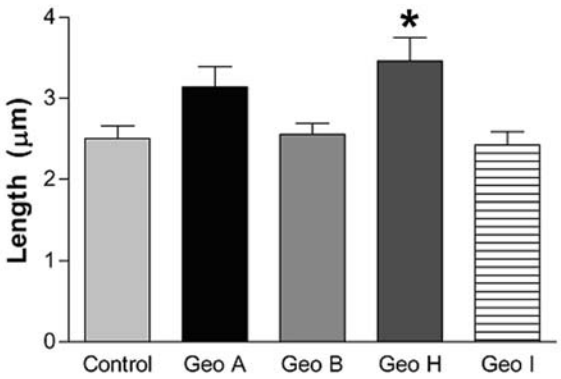

Figure 2 GJ length of HTC–Cx43–GFP cells, control and after treatments with the geodiamolides at a 200-nM concentration
Bars indicate means and S.E.M.; values were compared by ANOVA and Newman–Keuls multiple comparison test.

cells were treated concomitantly with Geo H and BFA, we also observed Cx43 accumulated in the cytoplasm and the absence of structures corresponding to GJ plaques at cell-to-cell contact areas (Figure 4).

4. Discussion

Several proteins can be involved with the life cycle of connexin (intracellular trafficking, connexon assembly and disassembly, insertion in the plasma membrane and degradation) including cytoskeleton proteins (Giepmans, 2004). Thus, the turnover of connexin can be affected by changes in the actin filament distribution pattern.

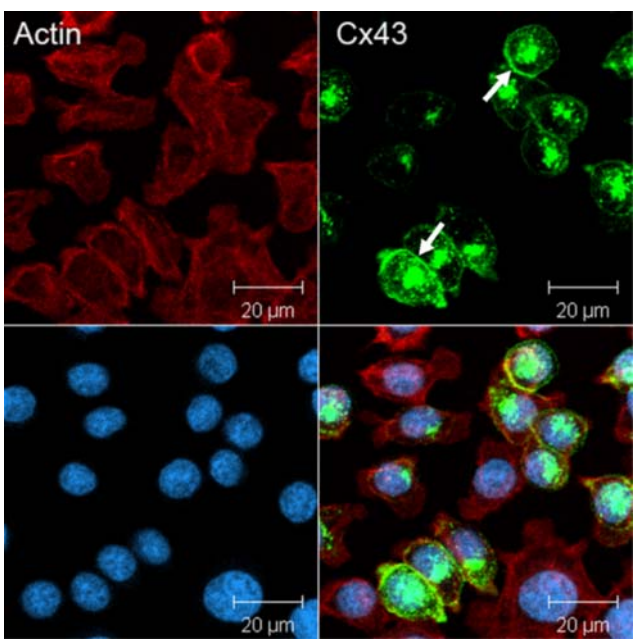

Figure 3 Laser scanning confocal microscopy images of HTC–Cx43–GFP cells treated with Geo H for 4 h (200 nM)
Microfilaments were stained with phalloidin–TRITC (red); Cx43 is shown in green. The GJ plaques are extensively evidenced on cell–cell contact areas (arrows). No alteration in microfilament distribution was observed. The nuclei were stained with TOPRO-3 (blue).

In the present work, we observed that geodiamolides A and H at low concentrations (100–200 nM) can increase the length of GJ plaques in rat hepatocarcinoma cells that express exogenous Cx43 (connexin 43) fused to GPF (HTC–Cx43–GFP cells). The effect of Geo H was higher than geodiamolide A suggesting that structural difference between β-tyrosine and alanine is not important in this case, but the halogen substituent *X* in the phenol ring of *N*-methyltyrosine moiety is crucial because the effect of geodiamolides H and A (X=I) is much more potent than geodiamolides B and I (X=Br). This structure–activity relationship was observed for these depsipeptides in our previous work describing their antiproliferative effect in breast cancer cells and sea urchin eggs. It is important to note that growth inhibition was consequent of the microfilaments disorganization (Rangel et al., 2006).

We also observed previously that Geo H promotes reversion of transformed phenotype in breast cancer cells (Hs578T) due to modification in the microfilaments distribution. However, Geo H did not affect the actin filaments organization in normal breast cells (MCF10A) (Freitas et al., 2008). Interestingly, in the present work, the effect promoted by Geo H on GJ plaques of HTC–Cx43–GFP cells was not accompanied by changes in the microfilaments distribution pattern. Actin filaments were disrupted only when higher Geo H (400 nM) concentration was used. In this case, the cells presented changes in the shape and did not form GJ channels.

The effect induced by Geo H at low concentrations on GJ channels could be related to stabilization of GJ plaques due to both (i) improvement in the connexon exportation or (ii) inhibition of the degradation pathway (Murray et al., 1997; Hervé et al., 2004). To uncouple events leading to GJ assembly from those related to GJ removal (Laird et al., 1995), we performed experiments where the cells were treated with Geo H in combination or not with fungal antibiotic BFA, a drug that disrupts the Golgi and protein trafficking to plasma membrane. In our system, BFA drastically reduced the GJ plaque formation, and the same response was obtained when the cells were treated with BFA and Geo H simultaneously. These data indicate that the peptide affects mainly the delivery pathway of Cx43 protein.

The phosphorylation state of Cx43 is an important event for its docking on to the plasma membrane. In a previous study, we reported that changes in original Cx43 phosphorylation profile of HTC cells can alter connexon exportation mechanism (Ionta et al., 2009). Thus, we cannot rule out a possible interaction of Geo H with specific kinase proteins. It has been demonstrated that PKA activation increases Cx43 movement to the plasma membrane and that CK1 activity also is involved in the assembly of Cx43 hemichannels into GJ plaques (Burghardt et al. 1995; TenBroek et al., 2001). Further studies are needed to understand molecular details about Geo H activity; however, we showed that the Cx43 exportation mechanism is improved when HTC–Cx43–GFP cells are treated with Geo H.

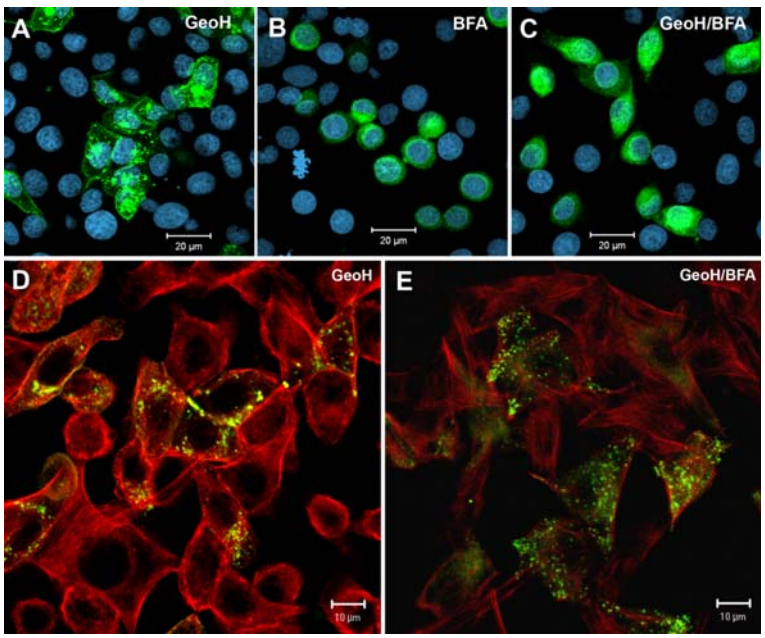

Figure 4 **Laser scanning confocal microscopy images of HTC–Cx43–GFP cells.**
(**A**) GeoH was used at 200 nM for 4 h; (**B**) BFA treatment inhibited the delivery pathway of Cx43 protein to membrane and (**C**) similar effect was observed when the culture was treated concomitantly with Geo H/BFA, the nuclei in blue. Microfilaments stained with phalloidin–TRITC (red) are shown in (**D**) 200-nM GeoH-treated cells and (**E**) BFA-/Geo H-treated cells.

5. Conclusions

The experiments performed with HTC–Cx43–GFP cells showed that geodiamolides increased the length of GJ plaques. Geo H was the peptide that exerted stronger effect, inducing long GJ plaques. Considering that GJ plaques were drastically reduced after simultaneous BFA and Geo H treatment, we conclude that Geo H increases the length of GJ plaques mainly because it improves the delivery pathway of Cx43 protein.

Author contribution

Glaucia Maria Machado-Santelli designed the study, analysed the fluorescent preparations, conceived the manuscript and wrote its final version. Raphael Adolpho Sant'Anna Ferreira and Sandra Cristina Pfister constructed the connexin–GFP vectors and performed cell transfections. Marisa Ionta and Marisa Rangel carried out the treatments, the cytological preparations, analysed the data and drafted the manuscript. Marisa Rangel isolated the geodiamolides. All the authors read and approved the final manuscript.

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